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TITLE: Characterizing the Dynamic Response of the Estrogen Receptor to Agonists and Antagonists by Multi-frequency Electron Spin Resonance Spin-Labeling

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14. ABSTRACT The overall objective of this project is to characterize the detailed structural and dynamic response of the estrogen receptor ligand binding domain (ER-LBD) to a variety of ligands ranging from strong estrogens to strong antiestrogens using electron spin labeling. The technical aims for the initial period involved developing site-directed spin-labeled mutants of the ER-LBD and synthesizing new spin-labeled ligands for the proposed studies. We have optimized cell culture production of ER-LBD and expressed a number of single and doubly labeled mutants. In addition, we have synthesized a new spin label for attachment to site-selected cysteine residues in this protein, and made substantial progress towards the synthesis of spin-labeled ligands with a range of agonist/antagonist activities. Initial electron spin resonance studies have established that the selected label locations will be acceptably sensitive to the type of ligand present.

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None provided

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Introduction

This proposal focuses on the key first steps in the estrogen response of breast cells, specifically the physical interaction between estrogen-like molecules and the ligand binding domain (LBD) of the ER. Although recent crystal structures of ER-LBD have implicated the C-terminal helix-12 (H12) of the ER in this response, X-ray analysis cannot characterize the dynamic behavior of H12 that is thought to play a major role in the tissue selectivity of the ER. By placing nitroxide spin labels at strategic points on the ER-LBD protein as well as on estrogenic ligands, we can map the dynamics and key distances in the complex over the entire range of ligand activities from estrogenic to antiestrogenic. This will afford the first characterization of the ER response under near physiological conditions, which will significantly aid the design of partially selective estrogen modulators for breast cancer therapies.

Progress Report

Specific aims for the first year involved synthesis of the necessary spin-labeled estrogen ligands, development and expression of single- and double- cysteine mutants of the ER-LBD for spin-labeling.

Task 1 *Synthesize a novel series of estrogenic probes with a nitroxide reporter group substituted at the 17α position and a short alkyl substituent at the 11β position to control the probe's activity (Months 1-24)*

We found that the disulfide linkage of the commercially available MTSL spin label was labile under the conditions required to maintain a stable form of the ER-LBD. We therefore devoted our initial synthetic effort to synthesize an iodomethyl spin label for this purpose (see Scheme 1 in Appendix) We find that the thioether linkage of this label stabilizes it and we expect it to provide a better reporter of protein dynamics as well.

We have made significant progress towards developing the novel estrogenic spin labels to be used in our work. For the proposed 11β-substituted antiestrogenic ligand, we have obtained the propargyl-substituted TEMPONE (see Scheme 2, Appendix). The immediate next step will be to couple this to an azide group at the 11β position of estrogen via a "click" (Huisgen 1,3 dipolar cycloaddition) reaction.

Our initial approach to synthesizing a brominated nitroxide for Stille coupling to the 17α position (see Scheme 3, Appendix) required some modifications due to the formation of an insoluble intermediate. We have now optimized the conditions (48% HBr with refluxing) that lead to completion of the reaction, and are presently preparing the spin-labeled moiety in sufficient quantities to carry out the final coupling reaction. The estrogen-based precursors needed for both the Stille and the click coupling reactions have been prepared and characterized (Scheme 4, Appendix) and we anticipate completing the synthesis of the first estrogen spin labels in the next 1-2 months.

Task 2**:** *Generate a series of site-directed spin-labeled mutants of the estrogen receptor α isoform (ERα) with labeling sites near the putative flexible Helix 12 region consisting of residues 538-548 (Months 1-24).*

We have optimized cell culture conditions and are now able to express ERα-LBD at considerably higher levels than reported in the literature (at least a factor of 3). Table 1 below summarizes our first year progress towards developing the range of ER-LBD mutants outlined in our proposal. Our first year goals were to prepare a construct with all active cysteines removed (M_0), two mutants with a single native cysteine (M_1 and M_2) and a mutant with a single label on H12 (M_8). These mutants have all been expressed at the levels necessary for EPR spectroscopy (*cf.* Table 1) and we have carried out initial spin-labeling EPR experiments on mutant M_1.

Table 1: Mutants for ER study (ERα-LBD)

	Mutation	**Description**	**Usage**	**Label sites**	**Status**
WT	none	wild type	standard/control binding assays	381+417+ 350	expressed
M_0	C381S/C417S/ C530S	Cys-free mutant	"blank" Cys-free mutant	none	expressed
M_1	C381S/C417S	hinge label	hinge dynamics	530	expressed labeled
M_2	C381S/C530S	protein body label	control for dynamics measurements	417	not constructed
M_3	M_1 + L539C	hinge+H_{12}	H_{12} distance measurement	530+539	not constructed
M_4	M_1 + L541C	hinge+H_{12}	H_{12} distance measurement	530+541	not constructed
M_5	M_1 + M543C	hinge+H_{12}	H_{12} distance measurement	530+543	expressed
M_6	M_0 + L539C	label H_{12}	H_{12} dynamics	539	not constructed
M_7	M_0 + L541C	label H_{12}	H_{12} dynamics	541	not constructed
M_8	M_0 + L543C	label H_{12}	H_{12} dynamics	543	expressed
M_9	C381S	hinge+body	H_{12} distance measurement	417+530	constructed

Task 3 *Characterize by EPR spectroscopy the specific structural differences that accompany binding of agonists and antagonists in complexes of spin-labeled estrogens to ER that is also spin-labeled. (Months 12-36)*

This task was scheduled to start after month 12 according to the original Statement of Work. We have carried out some preliminary 9 GHz EPR studies of mutant M_1 in the presence of estradiol and the antiestrogen RU39411 , which are shown in Figure 1 below. Although further adjustment of conditions will be required to optimize the EPR signal and eliminate signals from unattached label (sharp signals in Figure 1), the results already reveal lineshape changes (arrows) indicative of significant ligand-dependent dynamic changes in the H12 region. An in-depth analysis of the results utilizing our locally developed EPR lineshape analysis software is under way.

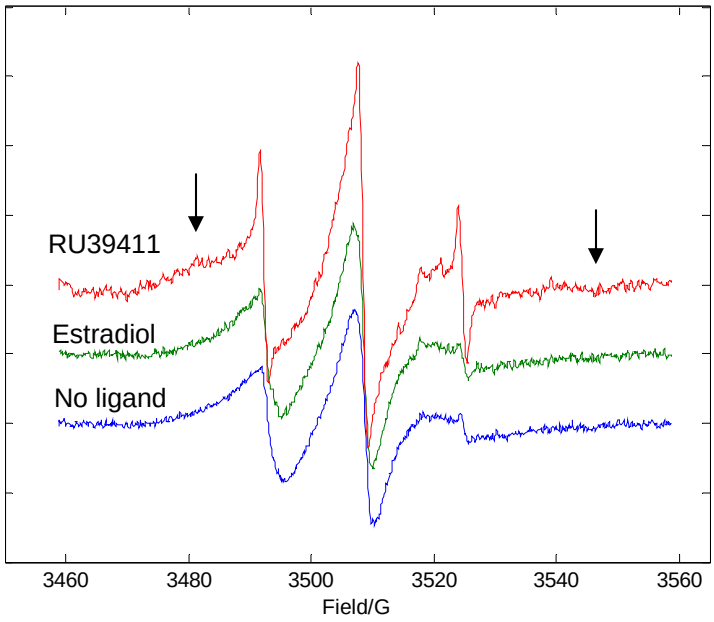


Figure 1: 9 GHz EPR spectra of ER mutant M1 spin-labeled with IMSL in the presence of no ligand (bottom), estradiol (middle) , and the antagonist RU39411 (top). Sharp lines in each spectrum are due to unbound label. The antagonist produces broad features in the spectrum (arrows) that indicate immobilization of the hinge region near H12.

3. Key Research Accomplishments

1. Developed improved synthesis for iodomethyl spin label
2. Prepared precursors needed for the final step in the synthesis of both agonist and antagonist estrogen-based spin labels.
3. Optimized expression of four key ER-LBD mutants for spin-labeling studies, including a mutant with the label introduced in the target helix-12 region
4. Demonstrated significant ligand-dependent dynamic changes in the hinge region between helix 12 and the body of the ER-LBD protein.

4. Reportable Outcomes

Our initial labeling experiments, including the synthesis of the IMSL spin label we have developed, will be reported at the International EPR Symposium of the August, 2007 Rocky Mountain Conference on Analytical Chemistry.

5. Conclusions

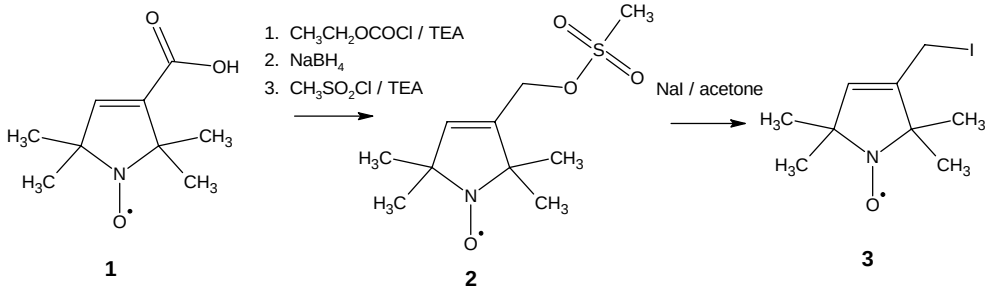
The project is proceeding on schedule according to the original Statement of Work. Tasks 1 and 2 (Synthesis of spin labeled estrogenic ligands and production of spin-labeled mutants) are scheduled to be completed by the end of Year 2. Task 3 is underway and has already provided preliminary information about ligand-dependent dynamics in the Helix 12 region of the Estrogen receptor.

References

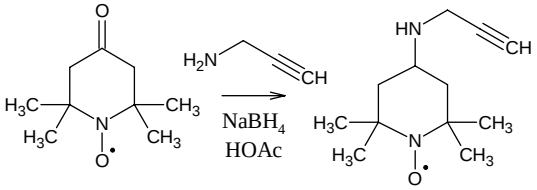
None.

Appendix

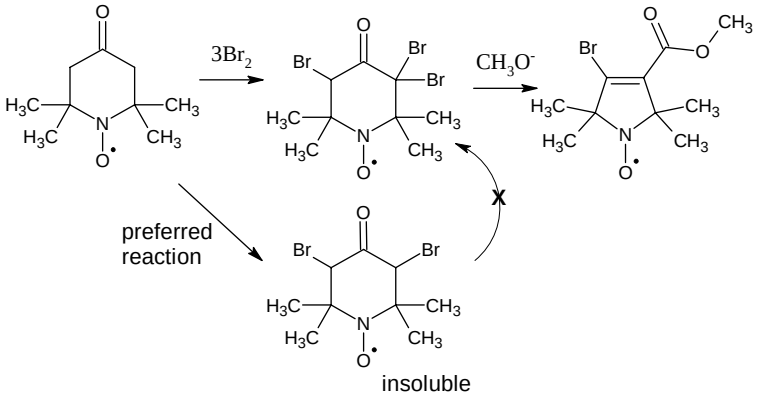
The following schemes depict reactions referred to in the body of the Progress Report.



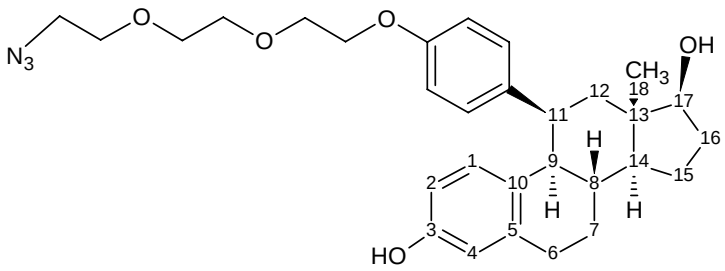
Scheme 1: Synthesis of iodomethyl spin label IMSL for labeling the ER protein

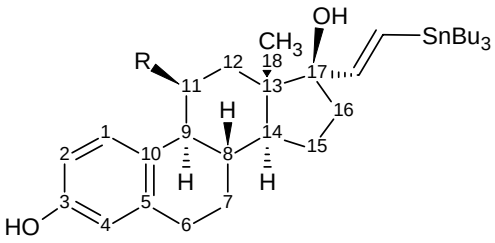


Scheme 2: Synthesis of Spin Probe 2 for attachment to antiestrogen steroid by "click" reaction.



Scheme 3: Synthesis of nitroxide moiety for attachment to estrogenic steroids by Stille reaction.





Scheme 4